Influence of the ELISA Format and the Hapten-Enzyme Conjugate on the Sensitivity of an Immunoassay for *s*-Triazine Herbicides Using Monoclonal Antibodies†

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A variety of formats for enzyme-linked immunosorbent assays have been examined for the analysis of environmental samples using a single monoclonal antibody raised to the herbicide atrazine. The most favorable combination of several ELISA formats and a variety of synthesized hapten-enzyme conjugates resulted in a highly sensitive enzyme immunoassay with 50% inhibition (I_{50}) at 0.25 μg/L and a detection limit of 0.03 μg/L. This assay is the most sensitive atrazine assay, based on monoclonal antibodies published so far, and can be performed in less than 1 h. All formats were based on the use of hapten-enzyme conjugates (enzyme tracers). Direct coating and precoating with goat anti-mouse antibodies or protein A were used. The formats using precoating steps turned out to be the most sensitive. Enzyme tracers based on two different atrazine and simazine haptens each with two different spacers (C_3 or C_6) were conjugated using either alkaline phosphatase or horseradish peroxidase. The atrazine derivative with the C_6 spacer arm conjugated to horseradish peroxidase provided highest sensitivity.

INTRODUCTION

Herbicides in the *s*-triazine class are considered prime indicator compounds for pesticide contamination in water and soil by the State of California and several U.S. federal agencies. The growing concern about the potential contamination of groundwater and drinking water and the implementation of the European Community (EC) directives necessitate rapid, inexpensive, and sensitive methods for high sample throughput analysis.

Enzyme-linked immunosorbent assays (ELISAs) have been demonstrated to be a fast screening method and a sensitive, quantitative analytical tool in pesticide (e.g., *s*-triazine) analysis (Bushway et al., 1989; Hammock, 1988; Jung et al., 1989; Schlaeppi et al., 1989; Wittmann and Hock, 1989, 1991).

The choice of the hapten for the preparation of immunogens largely dictates the properties of the raised antibodies. These antibodies determine the efficiency of the ELISA since the assay sensitivity is limited by the affinity of the antibody for the analyte (Tijssen, 1985).

For obtaining maximum performance from the antibody, a strategy for incubation (the ELISA format) of the antibody, enzyme tracer, and analyte (atrazine) that allows the use of low antibody and enzyme tracer concentrations in the competition step has to be chosen. In this way the analyte can compete strongly even if it is present only in low concentrations. The conjugated hapten should have an affinity to the antibody slightly smaller than that of the analyte to obtain optimum conditions for competition. Also, the catalytic activity of the hapten-enzyme conjugate (enzyme tracer) is of high importance.

A comparison of different ELISA formats based on the immobilization (coating) of either anti-atrazine antibodies, goat anti-mouse (trapping antibodies), or protein A to the polystyrene surface of the plate was performed. The format in which the anti-analyte antibodies are coated directly on the plates and which uses a competition between enzyme tracer and analyte is well established. Here an alternative format, using two sequential coating steps with anti-mouse and subsequently anti-analyte antibodies, is evaluated. The use of protein A, which binds specifically to the Fc region of the antibody, instead of an anti-mouse antibody to orient the antibodies to the competing reagents is described.

Previous work using various *s*-triazine-haptens (Goodrow et al., 1990) with different spacer arms for the synthesis of coating antigens has been published (Harrison et al., 1991b). In this study four different haptens, attached to the marker enzymes alkaline phosphatase (AP) and horseradish peroxidase (HRP), were applied in the ELISA. Additionally, HRP conjugated tracers, with different amounts of hapten, were compared. The most sensitive ELISA format was tested for ruggedness by the analysis of spiked water samples from different sources and with soil samples.

MATERIALS AND METHODS

Synthesis of Enzyme Tracers. Several enzyme tracers based on two different atrazine and simazine haptens (Goodrow et al., 1990) each with two different spacers were conjugated using either AP from bovine intestinal mucosa (Sigma, P 0405) or HRP from horseradish (Boehringer Mannheim, 814 393) (hapten-enzyme conjugates A-D; Figure 1). In each case the modified active ester method of Langone and Van Vunakis (1975) was used.

Alkaline Phosphatase Tracers. The carboxyl group of 3 μmol of the triazine derivatives was reacted with 15 μmol (1.7 mg) of *N*-hydroxysuccinimide (Sigma) and 30 μmol (6.2 mg) of dicyclohexylcarbodiimide (Aldrich) in 130 μL of dimethylformamide (DMF) and stirred for 4 h at room temperature and for another 12 h at 4 °C. After centrifuging, the clear supernatant, containing the active ester, was added slowly to 0.5 mg of AP in a solution of 260 μL Tris-buffered saline [tris(hydroxymethyl)-aminomethane, 50 mmol/L; $MgCl_2$, 1 mmol/L; NaCl, 1 mmol/L; pH 7.8 (TBS)] and DMF 1/1 (v/v). After 1 h, the DMF was diluted with another 200 μL of TBS buffer to create milder conditions for the enzyme. This mixture was allowed to react for 16 h at 4 °C. Afterward, the conjugates were purified from excess hapten by dialysis.

Horseradish Peroxidase Tracers. The preparation of the active ester was performed as described for the AP tracers. Active ester (130 μL) was added slowly to 2 mg of HRP in 3 mL of 0.13

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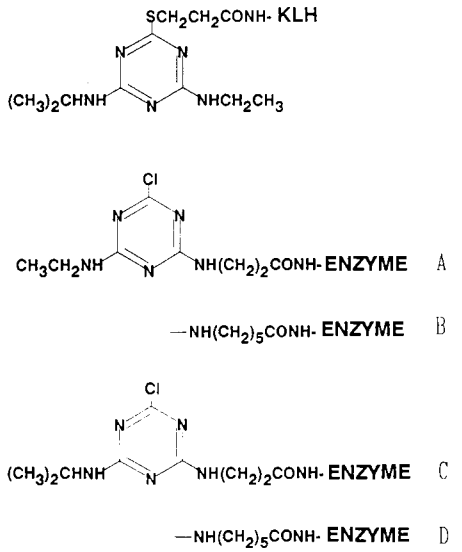


Figure 1. KLH conjugate and the hapten–enzyme conjugates. Two simazine tracers (A and B) and two atrazine tracers (C and D), each with a short C_3 or a long C_6 handle, were conjugated. The haptens were attached via their carboxyl groups to the amino groups of the enzymes. Additionally presented is the atrazine thiopropionic acid, which was coupled to hemocyanin from keyhole limpet (KLH) for immunization (Goodrow et al., 1990).

M $NaHCO_3$. The conjugates were dialyzed against 4 L of $NaHCO_3$ solution for 3 days with buffer changes twice a day. For testing the effects of different hapten/HRP ratios, various amounts of the activated hapten, containing an isopropyl and a hexanoic acid side chain [0.3 (for enzyme tracer 1), 0.6 (for enzyme tracer 2), 3 (for enzyme tracer 3), and 6 μmol (for enzyme tracer 4)], were reacted with constant amounts of HRP (2 mg) to make conjugate D (Figure 1) as described above. Both AP and HRP enzyme tracers were stored at −20 °C for up to several months after an equal volume of ethylene glycol was added to protect the solution from freezing.

ELISAs. The inhibition curves were run with dilutions of hybridoma culture fluid from monoclonal mouse anti-atrazine antibodies (AM7B2.1) from D. Schmidt and A. Karu, University of California, Berkeley (Karu et al., 1990). For coating, anti-atrazine antibodies, trapping antibodies (goat anti-mouse), or protein A was incubated in Nunc microtiter plates (4-42404) overnight at 4 °C in 0.5 M carbonate buffer (pH 9.6). If subsequent coatings were done (formats II and III), plates were washed, and then the next coating solution was added, incubated for 12 h, and washed another five times.

For running assays in formats I–III (see below), 50 μL of standard (stock solution, 1 mM in dimethyl sulfoxide) or sample and 50 μL of PBS-diluted enzyme tracer, both prepared in 0.2 M phosphate buffer in 8% saline supplemented with 0.1% (v/v) Tween 20 (PBST), were incubated on the coated plate for 15 min at room temperature. Plates were rinsed five times with PBST after each coating and after the competition step to remove the unbound immunoreactives. Standards were run in triplicate or quadruplicate.

Color development was obtained for HRP by adding 100 μL of substrate solution. The substrate solution was 200 μL of the chromogen tetramethylbenzidine (TMB) (6 mg in 1 mL of dimethyl sulfoxide) and 50 μL of 1% H_2O_2 in 12.5 mL of 0.1 M sodium acetate buffer (pH 5.5). The reaction was stopped after 15 min by adding 50 μL of 4 M H_2SO_4. The absorbances were measured at 450 nm. A solution of 100 μL of *p*-nitrophenyl phosphate (1 mg/mL in 1 M diethanolamine buffer, pH 9.8) was used for AP. The color was read after 1 h in a UV max reader (Molecular Devices) at 405 nm. All inhibition curves were calculated by the four-parameter logistic equation, with the color reaction being inversely proportional to the triazine concentration. The I_{50} represents the concentration of atrazine (or analogues) reducing the signal to 50% of the control (signal of zero concentration). The concentrations of the immunochemicals used in the individual ELISA formats were determined by two-dimensional titrations according to the method of Gee et al. (1988).

Four different formats were tested.

Format I (Direct Coating). The microtiter plates were coated directly with the anti-atrazine culture fluid (1:3200). The assay was performed as described before (HRP tracer 1:20000; AP tracer 1:1000).

Format II (Precoating with Goat Anti-Mouse IgG). The plates were coated first with the goat anti-mouse antibody (affinity-purified goat anti-mouse IgG and IgM; 1 mg/mL, Boehringer Mannheim; 605 24) (1:2000) and subsequently with the anti-atrazine antibody (1:3200), and the assay was performed as described above (HRP tracer 1:40000; AP tracer 1:1000).

Format III (Precoating with Protein A). The plates were coated with 1 μg/mL protein A from cell walls of *Staphylococcus aureus* (Cowan strains, Sigma; P 6650) and subsequently with the anti-atrazine antibodies (1:3200). The assay was performed as described above (HRP tracer 1:40000; AP tracer 1:1000).

Format IV (Separate Competition Step). Plates were coated with the goat anti-mouse antibody (1:3200). The competition step was done in a separate plate (Dynatech 001-012-9200) by adding 100 μL of the anti-atrazine antibody (1:320), 40 μL of standard solution, and 100 μL of enzyme tracer (1:10000). After a 30-min incubation, each 50 μL of this solution was added to coated Nunc microtiter plates. After another 30-min incubation, the plates were washed and the enzyme activity bound to the plate was measured.

Determination of Cross-Reactivities. The cross-reactivities (CR) were related to atrazine. The data was obtained from standard curves of the tested compounds and calculated according to the formula

$$\%\ \mathrm{CR} = \frac{\text{(hapten concn at 50\% } B/B_0\text{/ concn of the cross-reacting hapten at 50\% } B/B_0)}{} \times 100$$

Sample Applications. Samples were analyzed utilizing ELISA format II and HRP tracer D (Figure 1). For this purpose different water samples (tap water, water from San Francisco Bay, and water from Putah Creek, Davis) were spiked with atrazine, and the concentration was determined in the ELISA. In addition, soil samples [water leachates from 200 g of atrazine-spiked Delhi soil (loamy sand) leached with 100 mL of water in a column, provided by the California Department of Pesticide Regulation (CDPR)] were measured in the ELISA and the results compared to GC data (K. S. Goh, CDFA, Sacramento, CA, personal communication). Some of these samples were determined without dilution; some had to be diluted to adjust them to the measuring range of the test.

Soil samples (soil 1, mucky silt loam, organic matter 11.2%; soil 2, fine sandy loam, organic matter 1.4%) extracted with aqueous methanol (1 mL of solvent for each gram of soil) according to a slightly modified method of Muir and Baker (1978), without the described cleanup step using a cationic exchange column, were determined directly after dilution with PBS buffer to final concentrations of 1%, 5%, and 10% (v/v) methanolic extract in buffer. The methanol concentration of the standard solutions was adjusted to the concentrations of the samples to eliminate solvent effects.

RESULTS

Hapten/HRP Ratios. The comparison of the HRP enzyme tracers (conjugate D, Figure 1; format II) with different amounts of activated hapten did not show significant differences in the sensitivity of the assay [50% inhibition (I_{50})s from 0.3 to 0.6 μg/L for enzyme tracers 1 and 2 and from 0.2 to 0.5 μg/L for enzyme tracers 3 and 4]. The absorbances after the substrate incubation were up to 8 times higher using enzyme tracers 3 and 4 compared to enzyme tracers 1 and 2 (see Figure 2).

The solutions of enzyme tracers 3 and 4 could be diluted 1:40000 to reach absorbances of approximately 0.6 after 15 min. The enzyme tracers 1 and 2 could be diluted only 1:5000, reaching absorbances of about 0.4 after the same time of incubation. The higher dilution of hapten–enzyme conjugates probably caused the slightly higher sensitivities using enzyme tracers 3 and 4.

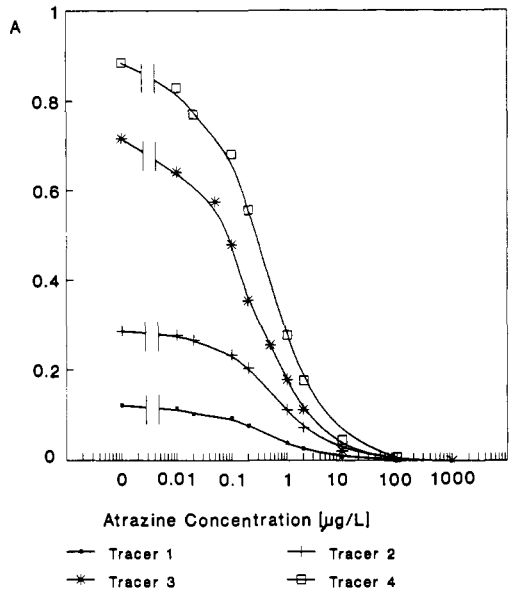


Figure 2. Comparison of HRP enzyme tracers (conjugate D in Figure 1) synthesized with various amounts of activated hapten. Each 2 mg of HRP was treated with 0.3 (tracer 1), 0.6 (tracer 2), 3 (tracer 3), and 6 μmol (tracer 4) of activated hapten. The different amounts of activated hapten used did not reveal any significant differences in the sensitivity of the assay; however, the absorbances were up to 8 times higher using tracers 3 and 4 compared to tracers 1 and 2.

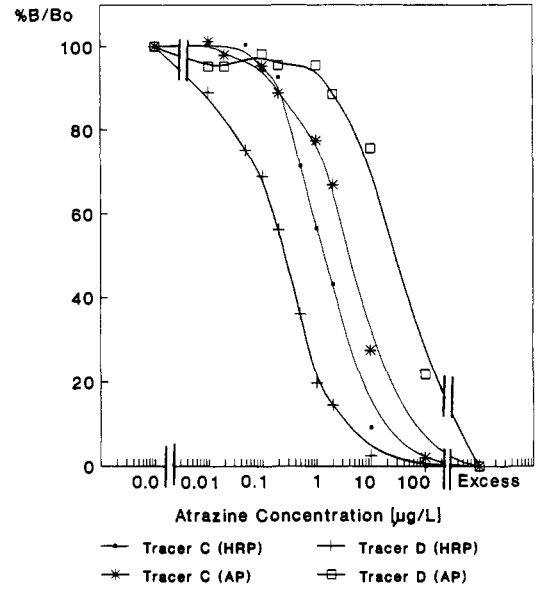


Figure 3. Standard curves for atrazine using enzyme tracers with different hapten spacer length (using format II). Four AP and HRP tracers (C and D; Figure 1) were compared. The longer spacer resulted in a more sensitive assay when conjugated to HRP and to a less sensitive assay when conjugated to AP. The shorter spacer arm resulted in more sensitive assays when conjugated to AP and in less sensitive assays when conjugated to HRP. In general more sensitive assays could be reached using HRP enzyme tracers (I_{50} at 0.24 μg/L using long spacer arms). The absorbances (A) were converted to $\%B/B_0$ values for normalization according to the following formula: $\%B/B_0 = [(A - A_{excess})/(A_{control} - A_{excess})] \times 100$.

Comparison of Different Haptens. Four different haptens (Figure 1) conjugated to HRP and AP resulted in different ELISA characteristics such as absorbance, sensitivity, and dilution. Figure 3 and Table I show the effects of the haptens used on the ELISAs. The haptens with C_6 spacer arms conjugated to HRP resulted in 3–5-fold increased sensitivity compared to the haptens with

Table I. Comparison of Eight Different Enzyme Tracers Synthesized from Four Different Haptens Conjugated to Each HRP and AP (Hapten Enzyme Conjugates A–D, Figure 1) and Applied in the ELISA (Format II)[a]

enzyme tracer	I_{50}	absorbance max	absorbance min	slope	tracer dil
A (HRP)	2.58 ± 1.03	0.6–0.8	<0.020	1.02 ± 0.12	1:2500
B (HRP)	1.83 ± 1.17	0.7–0.8	<0.010	1.05 ± 0.06	1:2500
C (HRP)	0.37 ± 0.11	0.3–0.4	<0.015	0.88 ± 0.07	1:10000
D (HRP)	0.24 ± 0.03	0.6–0.8	<0.005	0.99 ± 0.11	1:40000
A (AP)	3.55 ± 1.24	0.7–0.9	<0.013	1.10 ± 0.11	1:1000
B (AP)	2.17 ± 1.21	0.7–0.9	<0.004	1.13 ± 0.05	1:1000
C (AP)	12.07 ± 2.28	0.1–0.2	<0.010	1.01 ± 0.40	1:1000
D (AP)	29.33 ± 4.49	0.2–0.4	<0.008	0.96 ± 0.10	1:1000

[a] I_{50} data and slopes are presented as average values from 4–10 determinations with standard deviations.

Table II. Comparison of Different ELISA Formats Using Direct Coating or Precoating with either Goat Anti-Mouse or Protein A[a]

format	I_{50}	absorbance max	absorbance min	slope	tracer dil
		With HRP Conjugate			
I	0.48 ± 0.04	0.2–0.3	<0.008	1.01 ± 0.09	1:20000
II	0.29 ± 0.03	0.6–0.8	<0.006	0.99 ± 0.11	1:40000
III	0.24 ± 0.04	0.6–0.8	<0.005	0.89 ± 0.05	1:40000
IV	2.52 ± 0.51	0.5–0.8	<0.008	1.08 ± 0.10	1:10000
		With AP Conjugate			
I	2.33 ± 0.04	0.2–0.3	<0.007	1.23 ± 0.04	1:1000
II	2.17 ± 1.21	0.7–0.8	<0.005	1.13 ± 0.05	1:1000
III	1.82 ± 0.06	0.2–0.3	<0.004	1.18 ± 0.04	1:1000
IV	13.6 ± 3.4	0.6–0.7	<0.030	1.20 ± 0.10	1:1000

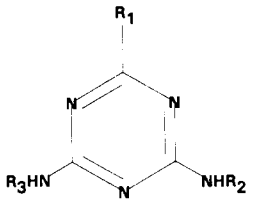
[a] All formats used the HRP conjugate D or the AP conjugate B. The values given for I_{50}s and the slopes correspond to the average and standard deviations of 4–10 different determinations. The structures of the conjugates are shown in Figure 1.

C_3 spacer arms. The reverse effect occurred by the use of the same haptens conjugated to AP. Here, the enzyme tracers with shorter spacers resulted in a higher sensitivity. The use of haptens with ethyl instead of isopropyl groups at the C5 position showed differences in sensitivity with the haptens having C_6 spacers conjugated to HRP. The HRP tracers could be diluted in ranges of 1:2500–1:40000, while dilutions of 1:1000 were needed for AP. The enzyme reaction for HRP could be stopped after 15 min; the AP reaction showed a sufficient absorption only after 60 min. In general, HRP tracers with the long spacer arms resulted in the most favorable assay conditions.

Comparison of Different Assay Formats. Assay formats greatly influence important parameters such as sensitivity, absorbance, and analysis time. The comparison of the different formats (using conjugate D of HRP and conjugate B of AP; Figure 1) is presented in Table II. The use of two coating steps (formats II and III) resulted in higher sensitivity and in about 3–4-fold increased absorptions compared to the format using one coating step (see Table II). The higher absorbances result in a more economical assay and slightly higher accuracy (the coefficients of variation among the triplicates or quadruplicates of the standard absorbances decreased from 5.0 to 4.4 for the AP conjugate and from 6.6 to 5.8 for the HRP conjugate using two coating steps).

The application of trapping antibodies was used in two different formats with the anti-atrazine antibodies competing in solution or immobilized on the antibody-activated polystyrene surface. Formats I–III, which involve the immobilization of anti-atrazine antibodies during the competition step, require a lower amount of antibodies and as a consequence also a lower amount of tracer and

Table III. Cross-Reactivities of Different Triazines to the Culture Fluid of AM7B2.1 Using Format II and the HRP Conjugate D as Enzyme Tracer[a]



compound	R_1	R_2	R_3	cross-reactivity based upon atrazine, %	I_{50}, μg/L
atrazine	Cl	CH_2CH_3	$CH(CH_3)_2$	100	0.24
simazine	Cl	CH_2CH_3	CH_2CH_3	32	0.75
atrazine mercapturate	*b*	CH_2CH_3	$CH(CH_3)_2$	386	0.06
ametryne	SCH_3	CH_2CH_3	$CH(CH_3)_2$	70	0.34
propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	147	0.16
terbutylazine	Cl	$C(CH_3)_3$	CH_2CH_3	20	1.20
hydroxyatrazine	OH	CH_2CH_3	$CH(CH_3)_2$	2	12
hydroxysimazine	OH	CH_2CH_3	CH_2CH_3	0	ni[c]
prometon	OCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	3	18
prometryn	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	17	1.41
terbutryn	SCH_3	CH_2CH_3	$CH(CH_3)_2$	19	1.26
simetryn	SCH_3	CH_2CH_3	CH_2CH_3	0	ni

[a] Presented are the I_{50}s and the corresponding cross-reactivities in percent based upon atrazine. [b] $SCH_2CH(NHCOCH_3)COOH$. [c] No inhibition.

analyte for an effective competition. These conditions probably cause the more than 10-fold increase of sensitivity for HRP and the more than 3-fold increase using the AP tracers in formats II and III.

The exchange of the goat anti-mouse antibodies with protein A for precoating to improve orientation of the antibodies (protein A binds specifically to the Fc region of the antibody and orients the Fab region for immunological reactions) resulted in slightly improved sensitivity using both HRP and AP tracers.

In general, the use of two sequential coating steps (formats II and III) provided the best sensitivity and reproducibility, which can be a consequence of high enzyme tracer dilutions. When the C_6–atrazine–HRP conjugate was used in these formats, an I_{50} of 0.24 μg/L was reached. The lower detection limit was approximately 0.03 μg/L. The average slope was determined to be 0.89. The absorption after the enzyme reaction (15 min) was between 0.6 and 0.8. The background (absorption of the standard solution of 100 μg/L compared to that of blank substrate solution) was always less than 0.005.

Cross-Reactivities. The antibodies showed class-specific properties with significant cross-reactivities for the related *s*-triazines simazine, propazine, ametryne, terbutryn, terbutylazine, and prometryn (Table III). Other related compounds or metabolites like prometon, hydroxyatrazine, or hydroxysimazine revealed no or low cross-reactivities. Atrazine mercapturate, which has a structure similar to the structure of atrazine thiopropionic acid, from which the hapten for immunizing was derived, cross-reacts almost 4-fold, compared to atrazine, but it is not likely to be found in water and soil samples.

Assay Application with Water and Soil Samples. The ELISAs were run with unspiked and atrazine-spiked water samples from different sources. None of the water samples showed any matrix effects (Table IV), even applied undiluted without any previous purification step.

The comparison of GC data [provided by K. S. Goh (CDPR)] and ELISA data for water-leached soil samples showed good correlation (Figure 4). Spiked and unspiked methanolic soil extracts from two different types of soil and also spiked and unspiked methanol dilutions were determined in the ELISA. Good recovery of the spikes could be reached for up to 10% methanol after the methanol concentration of the standards was adjusted to the concentration of the samples (see Table V). The data given in Table V further reveal the loss of sensitivity with higher concentrations of methanol. These data also

Table IV. Detection of Spiked Atrazine in Water Samples[a]

tap water		Putah Creek water		bay water	
spike	detected	spike	detected	spike	detected
0	<0.03	0	<0.03	0	<0.03
1.0	0.92 ± 0.15	0.5	0.42 ± 0.05	0.5	0.58 ± 0.07
1.0	1.23 ± 0.26	0.5	0.31 ± 0.02	0.5	0.45 ± 0.05
1.0	1.13 ± 0.30	0.5	0.36 ± 0.08	0.5	0.34 ± 0.06
5.0	3.87 ± 0.57	5.0	4.49 ± 0.61	5.0	7.53 ± 0.72
5.0	5.08 ± 1.13	5.0	5.16 ± 0.34	5.0	5.99 ± 0.39
5.0	4.07 ± 0.33	5.0	5.91 ± 1.15	5.0	5.65 ± 0.56

[a] All values are given in micrograms per liter. All samples were determined undiluted in triplicate or quadruplicate. Given are average values with standard deviations.

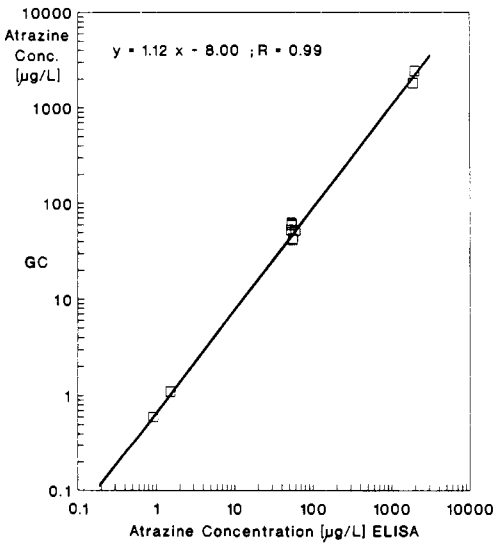


Figure 4. Correlation between GC and ELISA data for atrazine analysis of water-extracted soil samples (samples and GC data provided by CDPR). Samples were measured directly or diluted up to 50-fold to place them in the linear range of the ELISA.

Table V. Detection of Spiked Atrazine in Methanolic Soil Extracts[a]

spike	detected		
	1% methanol	5% methanol	10% methanol
	Soil 1		
0	<0.03	<0.06	<0.1
0.1	0.08 ± 0.03	0.12 ± 0.01	0.15 ± 0.03
0.5	0.54 ± 0.04	0.56 ± 0.12	0.65 ± 0.07
1.0	1.07 ± 0.02	1.19 ± 0.10	1.21 ± 0.17
	Soil 2		
0	<0.03	0.12 ± 0.06	0.31 ± 0.08
0.1	0.12 ± 0.01	0.19 ± 0.03	0.34 ± 0.14
0.5	0.52 ± 0.03	0.70 ± 0.12	0.76 ± 0.13
1.0	1.04 ± 0.03	1.38 ± 0.20	1.18 ± 0.07
	Methanol		
0	<0.03	<0.06	<0.1
0.1	0.08 ± 0.02	0.11 ± 0.02	0.10 ± 0.03
0.5	0.55 ± 0.04	0.65 ± 0.03	0.59 ± 0.07
1.0	1.03 ± 0.02	1.17 ± 0.15	1.12 ± 0.12

[a] All values are given in micrograms per liter. The samples were determined in 1%, 5%, and 10% methanolic soil extract in PBS buffer. The standards were adjusted to the methanol concentration of the samples. The samples were determined in triplicate or quadruplicate. Given are average values with standard deviations. As a control, the spikes were also determined in methanol solutions.

indicate that this format is sufficiently rugged to analyze real environmental samples. It must be cautioned that with any analytical technique different extraction techniques sometimes must be used with different soil types. It also is possible that other soils may yield interfering materials.

CONCLUSIONS

The chemical structure that links a hapten to a biopolymer is referred to as a handle. A common misconception is that recognition of these handles can be ignored if a monoclonal antibody is used. The studies in the triazine field illustrate that differential recognition of haptens with different handles usually is important when small haptens are studied. This brings up the possibility of improving even a monoclonal-based assay by systematic variation in the chemistry and position of handles.

With ELISA formats that use hapten enzyme tracers, the enzyme is a key reagent in the development of sensitive immunoassays. In this assay the HRP tracers showed higher sensitivity and, in addition, the enzyme reaction could be stopped after 15 min due to the high substrate turnover and sensitive color reaction with TMB. The same observation was made by Wittmann and Hock (1989) and Wüst et al. (1990).

All haptens used in this study had positional heterology (Harrison et al., 1991a). The length of the hapten spacers attached to the enzyme influenced the sensitivity of the subsequent assay strongly. The favorable use of longer spacers for HRP and shorter spacers for AP is surprising. The reason for the inverse effects could be a result of the different molecular weights of the enzymes (44 000 for HRP; 160 000 for AP) or of different hapten presentation as a consequence of different protein folding and steric conditions of the enzymes used. The results presented here emphasize the importance of examination of a variety of different haptens for conjugation to obtain high assay sensitivity, even when using monoclonal antibodies.

The active esters are expected to react largely with the ϵ-amino group of lysines and the N-terminal amino acids of the protein. The likelihood of reaction with any amine is a statistical phenomenon determined by many variables which are difficult to predict. For example, certainly the hydrophobicity of the medium and other reaction conditions will influence the local environment and thus the reactivity of different amines. Also, the properties of the activated hapten are likely to vary. Careful control of reaction conditions is likely to lead to reproducible conjugate synthesis. It is questionable if the very detailed protein modeling needed to improve our predictive ability will be cost effective. However, these data do illustrate the potential of using site-directed mutagenesis coupled with structural biochemistry to design improved enzyme tracers.

Of all formats used here those using two subsequent coating steps turned out to be the best. Both the anti-mouse IgG and the protein A used in a first coating step resulted in sensitive assays, relative high absorptions after short substrate incubations, and high reproducibility. The reasons for these effects are probably the low concentrations of antibodies and extremely high dilutions of enzyme tracers which could be used under the described conditions. Direct coating of the anti-atrazine antibodies to the plates also provided good results, but longer substrate incubation was necessary for equal color development, the sensitivity decreased slightly, and the plate-to-plate variation was larger than in other tested formats. The competition step using the anti-analyte antibodies in solution resulted in significantly lower sensitivity than with the antibodies immobilized on the plate. This is probably a consequence of the higher concentrations of anti-atrazine antibodies available in solution compared to the immobilized antibodies during the competition step. For the subsequent enzyme reaction, only the antibody–tracer complexes, trapped by the immobilized goat anti-mouse antibody, are available. In that way the concentrations of tracer and atrazine necessary for an effective competition with the antibodies in solution are greater than the concentrations required using a reduced amount of immobilized antibodies after the washing step.

All formats had low background absorptions. The lowest background relative to the maximum absorbance was found for formats II and III. The precoating makes blocking steps after coating (e.g., with BSA) unnecessary. The slopes in the linear range were quite constant when both different formats and different enzyme tracers were compared.

Monoclonal s-triazine assays have been published by Giersch and Hock (1990) with especially high sensitivities for prometryn and terbutryn and by Schlaeppi et al. (1989), who focused on atrazine and hydroxyatrazine. The format here described (formats II and III) using the HRP conjugate provided the most sensitive ELISAs for atrazine, simazine, and propazine based on monoclonal antibodies published so far. On the basis of the data presented here, it is very possible that our assay and the assay of the above workers could be modified by a systematic testing of different haptens on the enzyme tracer or coating antigen while using the same monoclonal antibody. These modifications could yield changes in both sensitivity and specificity of the resulting assays.

The described assay has sufficient sensitivity for direct applications to environmental samples, without any previous concentration steps, and no matrix effects have been observed, even when the analysis was done without previous dilution. Determination of atrazine and simazine in water and soil using the same antibodies is described by Lucas et al. (1991). The high cross-reactivities with simazine, ametryne, and propazine offer the possibility of a broad triazine screening test but limit the possibilities for a single-compound analysis. However, other hapten

antibody combinations in our library allow the development of assays that are class, family, or compound selective.

There are numerous methods in which antibodies can be used, with RIAs and ELISAs being the most well-known. Once a good poly- or monoclonal antibody is developed, it can be employed in a variety of formats, each with its unique set of relative advantages and limitations. Even within the ELISA method there are many variations possible in format. The formats described here represent a compromise of conditions yielding a rapid, highly sensitive, and reproducible assay that appears to be rugged and adaptable for field use. However, the conditions described also provide baseline information to apply this assay to a variety of other ELISA-based formats ranging from dipstick technology to biosensors.

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Registry No. Atrazine, 1912-24-9; water, 7732-18-5; atrazine mercapturate, 138722-96-0; ametryne, 834-12-8; propazine, 139-40-2; terbutylazine, 5915-41-3; hydroxyatrazine, 2163-68-0; hydroxysimazine, 2599-11-3; prometon, 1610-18-0; prometryn, 7287-19-6; terbutryn, 886-50-0; simetryn, 1014-70-6; simazine, 122-34-9.